Chronic Olanzapine or Fluoxetine Administration Increases Cell Proliferation in Hippocampus and Prefrontal Cortex of Adult Rat

Masafumi Kodama, Takashi Fujioka, and Ronald S. Duman

Background: *There has been increasing evidence that atypical antipsychotics are effective in the treatment of mood disorders or for augmenting 5-hydroxytryptamine selective reuptake inhibitors for treatment-resistant depression.*

Methods: *Upregulation of neurogenesis in the adult hippocampus is a marker of antidepressant activity, and the present study investigated the influence of the atypical antipsychotic drug olanzapine on cell proliferation in the hippocampus of adult rat. The regulation of cell proliferation in the prelimbic cortex of adult rat was also examined.*

Results: *Chronic (21 days) olanzapine administration increased the number of newborn cells in the dentate gyrus of the hippocampus to the same extent as fluoxetine. Olanzapine or fluoxetine treatment also increased the number of proliferating cells in the prelimbic cortex. In contrast, there was no effect of either drug in the subventricular zone or primary motor cortex, and there was a trend for an increase in the striatum. Subchronic (7 days) administration of olanzapine had no effect on cell proliferation in hippocampus or prelimbic cortex, consistent with the time course for the effect of fluoxetine and the therapeutic actions of antidepressant treatment. The combination of olanzapine plus fluoxetine did not result in a greater induction of cell proliferation in either brain region. Analysis of the cell phenotype demonstrated that approximately 20% of the newborn cells in the prelimbic cortex differentiated into endothelial cells but not neurons, in contrast to the dentate gyrus, where most newborn cells differentiated into neurons.*

Conclusions: *The results demonstrate that antidepressant or atypical antipsychotic medications can increase the proliferation of glia in limbic brain structures, an effect that could reverse the loss of glia that has been observed in depressed patients.*

Key Words: Antidepressant, atypical antipsychotic, endothelial, mood disorder, neurogenesis

Depression is one of the most debilitating and common psychiatric diseases and is reported to have a life-time expectancy of up to 20% (Blazer 2000). Several different types of treatment are commonly used, including chemical antidepressants, electroconvulsive seizures, and behavioral therapy, that result in successful treatment of approximately 80% of patients; however, there is still a significant number of patients who are not responsive to any of the currently available treatments, and those who do respond often have to wait several weeks or months to experience a therapeutic response.

For those who derive little benefit from conventional antidepressant strategies, novel treatments are being developed. Several clinical studies have reported that atypical antipsychotics, such as olanzapine, risperidone, quetiapine, and clozapine, are effective for the treatment of bipolar disorder, as well as for unipolar depression, particularly in depression with psychotic features (Ghaemi and Goodwin 1999; Rothchild 2003; Sajatovic et al 2002). Furthermore, augmenting an antidepressant by addition of an atypical antipsychotic has produced striking results (Hirose and Ashby 2002; Ostroff and Nelson 1999; Shelton 2003; Shelton et al 2001). Shelton et al's double-blind study of depressed patients without psychotic symptoms who did not respond to a 5-hydroxytryptamine (5-HT, serotonin) selective reuptake inhibitor (SSRI) demonstrates that the addition of an atypical antipsychotic (i.e., fluoxetine plus olanzapine) produced an effect in as little as 1 week that was sustained over the 8-week treatment period (Shelton et al 2001). Atypical but not typical antipsychotics are also reported to have antidepressant effects in a behavioral model of depression (Weiner et al 2003).

The mechanisms underlying the antidepressant and/or augmenting effects of atypical antipsychotics are still unclear. Studies of neurotransmitter release with microdialysis have demonstrated that acute olanzapine significantly increases both dopamine and norepinephrine levels in rat prefrontal cortex, nucleus accumbens, and striatum, and the combination of olanzapine plus fluoxetine produces a greater increase in levels of dopamine and norepinephrine in the rat prefrontal cortex than fluoxetine alone (Kuroki et al 1999; Li et al 1998; Zhang et al 2000); however, the adaptations that occur in response to repeated drug treatment and that underlie the long-term actions of atypical antipsychotic drugs have not been determined.

Recent studies demonstrate that chronic but not acute antidepressant treatment increases levels of neurogenesis in the adult hippocampus (Czeh et al 2001; Madsen et al 2000; Malberg et al 2000; Manev et al 2001; see Duman 2004). Moreover, blockade of neurogenesis with either a mutant mouse approach or by irradiation blocks the effects of antidepressant treatment in behavioral models (Santarelli et al 2003). The relevance of neurogenesis to stress-related mood disorders is further supported by studies demonstrating that stress or corticosterone treatment decreases neurogenesis (Czeh et al 2001; Gould et al 1997; McEwen 1999). Finally, antidepressant treatment reverses or blocks the effects of stress on adult neurogenesis (Czeh et al 2001; Malberg and Duman 2003; van der Hart et al 2002).

The possibility that decreased neurogenesis as well as atrophy or remodeling of other hippocampal neurons is relevant to the pathophysiology of depression (see Duman 2004; McEwen 1999) is supported by clinical studies demonstrating that the volume of the hippocampus is decreased in patients with depression or posttraumatic stress disorder (PTSD) (Bremner et al 1995; Manji

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et al 2001; Sheline et al 1996). Post hoc and longitudinal studies also demonstrate that antidepressant treatment blocks or even reverses the atrophy of hippocampus in patients with depression or PTSD (Sheehan et al 2003; Vermetten et al 2003). Although there are insufficient data to know whether altered neurogenesis accounts for the changes observed in depressed or PTSD patients, the results are consistent with the ability of stress and antidepressant treatment to regulate adult neurogenesis in the hippocampus.

In addition to alterations of the hippocampus, brain imaging and postmortem studies demonstrate a decrease in the volume of subregions of the prefrontal cortex (Manji et al 2001; Nestler et al 2002). Moreover, morphometric analysis of postmortem brain samples demonstrates a reduction in neuronal body size and number of glia in subgenual prefrontal cortex, rostral orbitofrontal cortex, and cingulate cortex (Cotter et al 2001; Ongur et al 1998; Rajkowska et al 1999). Because neurogenesis in adult brain is observed primarily in selected regions, including the subventricular zone and the hippocampus, it probably does not contribute to the altered volumetric changes observed in cortical regions of depressed patients; however, it is possible that antidepressant treatment influences the proliferation of nonneuronal cells, such as glia.

The current study was undertaken to test the hypothesis that an atypical antipsychotic drug, olanzapine, can increase neurogenesis in the adult rodent hippocampus or augment the effects of fluoxetine. In addition, the influence of both olanzapine and fluoxetine on cell proliferation in the prelimbic brain region was determined.

Methods and Materials

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, Massachusetts) weighing 250–300 g were housed under a 12-hour light/12-hour dark cycle (lights on at 7:00 AM, lights off at 7:00 PM) and at constant temperature (25°C) and humidity and allowed free access to food and water. Animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Yale University Animal Care and Use Committee.

Drug Treatment Protocols

For chronic drug treatments, rats were administered fluoxetine (5 mg/kg/day) or saline by intraperitoneal (IP) injection once daily and olanzapine (.5 or 2 mg/kg/day) or vehicle in the drinking water for 21 days (vehicle-treated control, fluoxetine, and olanzapine alone at either .5 or 2 mg/kg/day) plus the combination of fluoxetine and olanzapine (.5 mg/kg) (see Figure 1). For combination treatment, a dose of .5 mg/kg/day olanzapine was chosen because fluoxetine is known to interfere with the metabolism of olanzapine and raise the blood levels by up to 4–6 times (F. Bymaster, Eli Lilly and Company, personal communication). Olanzapine was dissolved in .01 N hydrochloric acid (HCl), then adjusted back to pH 6 with 1 N sodium hydroxide to make the stock solution of 3 mg/mL concentration. The same amount of vehicle solution was added to the water for the control animals. Fluid intake was measured three times per week, and drinking bottles were replenished with fresh drug solution. There were no differences in fluid intake among the treatment groups. For subchronic treatment, drugs were administered exactly the same way but for a total period of 7 days.

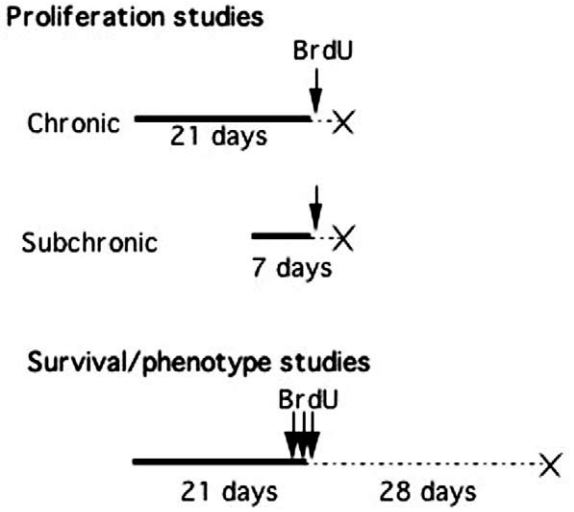

Figure 1. Time schedule for drug and bromodeoxyuridine (BrdU) administration. For the studies of cell proliferation, animals were administered fluoxetine or olanzapine for 21 (chronic) or 7 (subchronic) days. Three hours after the last drug administration, animals were administered BrdU (150 mg/kg) and killed (X) 24 hours later. For the survival and differentiation study, animals were administered BrdU (100 mg/kg) once daily for the last 3 days of the 21-day drug treatment and then allowed to survive for 28 days after the last BrdU injection.

Bromodeoxyuridine Administration

For proliferation studies, rats were administered 150 mg/kg bromodeoxyuridine (BrdU; Sigma, St. Louis, Missouri) by IP injection 3 hours after the last drug injection and/or removal of the drinking bottles. Twenty-four hours after the BrdU injection, rats were anesthetized by 400 mg/kg chloral hydrate and transcardially perfused with .1 mol/L cold phosphate-buffered saline (PBS) for 5 min, followed by 4% cold paraformaldehyde plus PBS for 20 min. For the survival and differentiation study, rats were administered 100 mg/kg BrdU injection once daily each of the last 3 days of the 21-day drug treatment and then allowed to survive for 28 days after the last BrdU injection. Animals were then anesthetized and perfused exactly as described above.

Immunohistochemistry

After perfusion, all brains were postfixed overnight in 4% paraformaldehyde in PBS at 4°C and stored in 30% sucrose PBS at 4°C. Serial sections of the brains (40-μm thickness) were cut on a freezing microtome, and sections were stored in PBS. Free-floating sections were subjected to deoxyribonucleic acid denaturation by incubation for 2 hours in 50% formamide/2× standard saline citrate (SSC) at 65°C, followed by a 2× SSC rinse. Sections were then incubated for 30 min in 2 N HCl, PBS, and then 10 min in .1 mol/L boric acid, pH 8.5. Sections were incubated for 30 min in 3% hydrogen peroxide to eliminate endogenous peroxidases. After washing in PBS twice, they were incubated with blocking buffer (3% normal horse serum in .3% Triton X-100, PBS) for 30 min. Then, cells were incubated with mouse anti-BrdU (1:100; Becton Dickinson, San Jose, California) with blocking buffer overnight at 4°C. Sections were then incubated for 1 hour with biotinylated horse antimouse antibody (Vector Laboratories, Burlingame, California) with blocking buffer, followed by amplification with an avidin-biotin complex (Vector Laboratories), and cells were visualized with diaminobenzadine with a light microscope (Vector Laboratories).

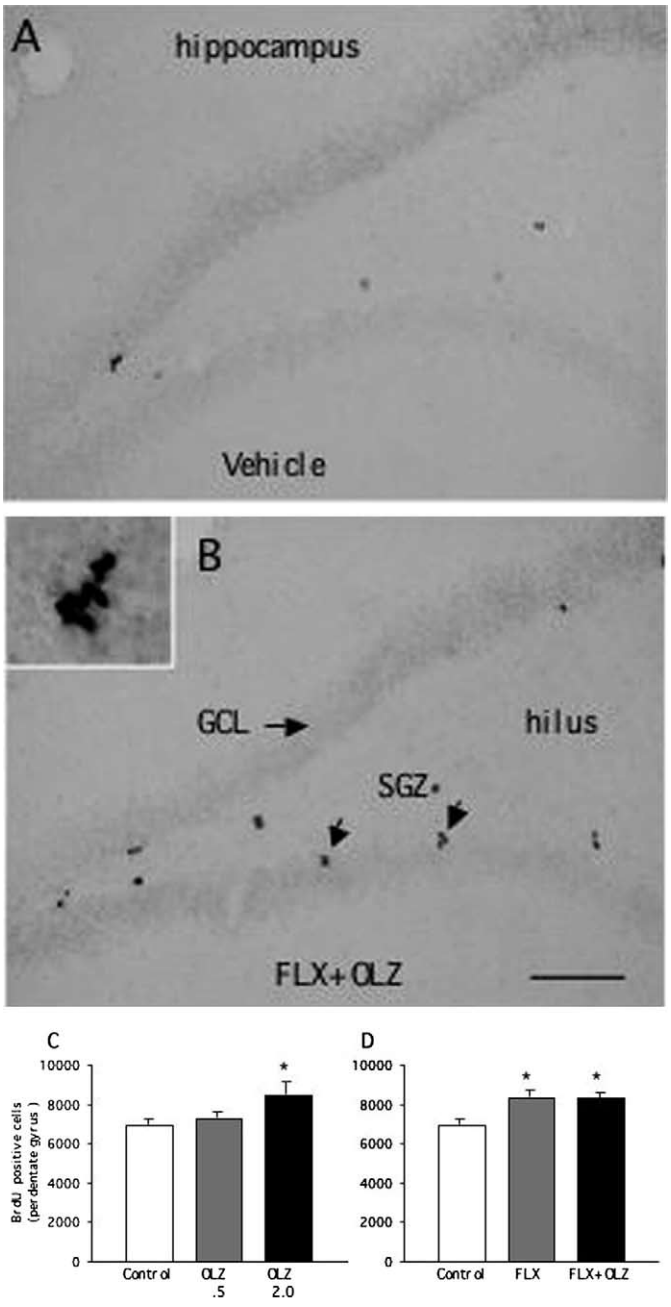

Figure 2. Chronic olanzapine (OLZ) or fluoxetine (FLX) administration increases the number of bromodeoxyuridine (BrdU)-labeled cells in the dentate gyrus of adult hippocampus. Rats were administered OLZ (.5 or 2.0 mg/kg), FLX (5 mg/kg), or the combination of OLZ + FLX (.5 and 5 mg/kg, respectively) once daily for 21 days. Rats were administered BrdU (150 mg/kg) 3 hours after the last drug treatment and killed 24 hours later for BrdU immunohistochemical analysis. Upper panels show representative BrdU-labeled sections from **(A)** control and **(B)** OLZ + FLX-treated animals. The granule cell layer (GCL), subgranular zone (SGZ), and hilus are indicated. The photomicrograph **insert in B** shows a higher magnification of a group of closely apposed BrdU-positive cells. Scale bar = 100 μm. The number of BrdU-positive cells in the dentate gyrus was determined with a stereologic procedure, and the results are shown in the lower panels. **(C)** Effects of different doses of OLZ. **(D)** Effects of FLX and the combination of OLZ + FLX. Values are expressed as mean ± SEM. Results were subjected to statistical analysis. *$p < .05$ (analysis of variance and Fisher's post hoc test).

For double and triple labeling, brain sections were incubated for 30 min with 2 N HCl, PBS. After washing twice in PBS, sections were incubated in blocking buffer (3% normal goat serum, .3% Triton X-100, PBS). Sections were then incubated for 2 days at 4°C with rat anti-BrdU (1:100; Accurate; Harlan Olac, Bicester, United Kingdom) and one or two of the following: mouse antineuronal nuclei (NeuN) (1:200; Chemicon, Temecula, California), rabbit anti-S100β (1:100; Dako, Glostrup, Denmark), mouse anti-O4 (1:200; Chemicon), and/or mouse antirat endothelial cell antigen-1 (RECA-1) (1:500; Serotec, Oxford, United Kingdom). The secondary antibodies were Alexa Fluor 488 goat antimouse (1:200), Alexa Fluor 546 goat antirat (1:200), and Alexa Fluor 633 goat antirabbit (1:200) (Molecular Probes, Eugene, Oregon) and were applied for 1 hour and visualized with confocal Z-plane sectioning by a Zeiss LSM 510 (Carl Zeiss, Jena, Germany).

Quantitation of BrdU Labeling

An experimenter blinded to the identity of the sections counted BrdU-labeled cells. To distinguish single cells within clusters, all counts were performed at 400× and 1000× under a light microscope (Olympus CH30; Olympus, Tokyo, Japan). For cell counting in the dentate gyrus, every 10th section throughout the whole hippocampus was processed for BrdU immunohistochemistry. A cell was counted as being in the subgranular zone of the dentate gyrus if it was touching or in the subgranular zone, which was defined as the cell layer located between the granule cell layer and the hilus. For other brain regions, every sixth section was collected and immunostained through the following brain positions: from 3.70 mm to 2.20 mm from Bregma for prelimbic cortex and primary motor cortex and from 1.20 mm to −.26 mm from Bregma for subventricular zone and striatum (Paxinos and Watson 1997) (Figures 3A, 4A). This method resulted in at least six sections for every brain region examined. For cell counting in the prelimbic cortex, primary motor cortex, and striatum, a 1 × 1-mm square scale was set on a specific region. All positive cells within the square scale were counted under high magnification. Cell counting for the subventricular zone was performed with a .1 × .1-mm square scale placed on the lateral wall of the lateral ventricle, owing to the small size and relatively large number of labeled cells in this region.

For double and triple labeling, slices were analyzed on a confocal microscope (Zeiss Axiovert LSM510). At least 50 (dentate gyrus) or 25 (prelimbic cortex) BrdU-positive cells per animal were analyzed with Z-plane sectioning (1-μm steps) to confirm the colocalization of both BrdU and the phenotypic markers for neurons (NeuN), glia (S100β), oligodendrocites (04), or endothelial cells (RECA-1).

Statistical Analysis

The differences among groups were analyzed by analysis of variance (ANOVA), followed by the Fisher's protected least significant difference (PLSD). The level of statistical significance was set at $p < .05$.

Results

Chronic Administration of Olanzapine or Fluoxetine Increases Cell Proliferation in the Hippocampus

Bromodeoxyuridine was administered to control or drug-treated animals, and 24 hours later rats were anesthetized and perfused for immunohistochemical analysis. Representative sections of BrdU immunohistochemistry for the dentate gyrus are shown in Figure 2A and B. A number of BrdU-positive cells are

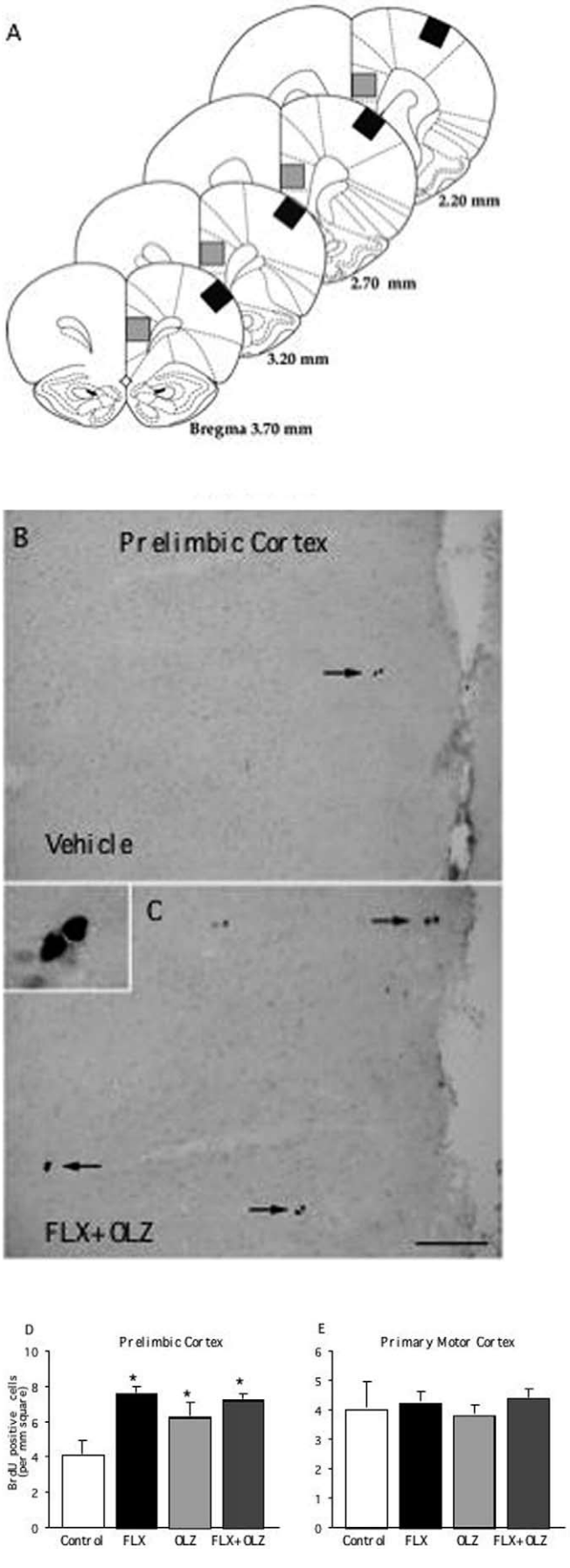

Figure 3. Chronic administration of olanzapine (OLZ) or fluoxetine (FLX) increases bromodeoxyuridine (BrdU)-labeled cells in the prelimbic cortex of rat brain. Rats were administered FLX (5 mg/kg), OLZ (2.0 mg/kg), or the combination of FLX + OLZ (5.0 and .5 mg/kg, respectively) for 21 days. Rats were then administered BrdU (150 mg/kg) 3 hours after the last drug treatment and killed 24 hours later for BrdU immunohistochemistry. **(A)** Cortical regions of interest on a series of plates from Paxinos and Watson 1997. The gray and black squares display 1 × 1-mm region for prelimbic cortex and primary motor cortex, respectively. **(B, C)** Representative BrdU-labeled sections of the prelimbic cortex from **(B)** control and **(C)** OLZ + FLX. Arrows indicate BrdU-labeled cells. The photomicrograph insert in **C** shows a high-power magnification of BrdU-labeled cells. Scale bar = 100 μm. The number of BrdU-positive cells was determined by stereologic analysis throughout the regions indicated in **A**, and the results are expressed as the number of cells per square millimeter for **(D)** prelimbic and **(E)** primary motor cortex. Values are expressed as mean ± SEM. Results were subjected to statistical analysis. *$p < .05$ (analysis of variance and Fisher's post hoc test).

seen along the subgranular zone. At this or earlier time points, BrdU-positive cells are often observed in clusters (see insert, Figure 2B).

The effect of low and high doses of olanzapine administered for 21 days on the cell proliferation in the dentate gyrus is shown in Figure 2C. The high dose (2 mg/kg) of olanzapine significantly increased the number of positive cells (reported throughout as mean ± SEM) in the dentate gyrus compared with the control group (p = .0139, Fisher's PLSD), whereas the low dose (.5 mg/kg) had no significant effect [control, 6910 ± 300; olanzapine .5 mg/kg, 7270 ± 260; olanzapine 2.0 mg/kg, 8440 ± 650; n = 10, 6, and 6, respectively; $F(2,19) = 3.757$; p = .0422]. The effect of olanzapine plus fluoxetine, as well as fluoxetine alone on proliferation in the dentate gyrus is shown in Figure 2D [control, 6910 ± 300; fluoxetine 5 mg/kg, 8340 ± 302; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 8260 ± 280; n = 10, 11, and 10, respectively; $F(2,28) = 7.053$; p = .0033]. Fluoxetine alone increased cell proliferation in the dentate gyrus (p = .0021, Fisher's PLSD), which is consistent with previous reports (Malberg et al 2000; Manev et al 2001; Santarelli et al 2003). Coadministration also significantly increased the number of BrdU-positive cells compared with the control group (p = .0040, Fisher's PLSD); however, there was no effect of the combination of fluoxetine plus olanzapine on cell number relative to fluoxetine alone.

Chronic Administration of Olanzapine or Fluoxetine Increases Cell Proliferation in the Prelimbic Cortex

Representative sections of BrdU immunohistochemistry of the prelimbic cortex are shown in Figures 3B and C. Figure 3A demonstrates the regions of prelimbic and primary motor cortex where BrdU-positive cells were quantified (Paxinos and Watson 1997). The density of BrdU-positive cells in these regions is much lower than in the subgranular zone of the dentate gyrus. It is often seen that two BrdU-positive cells are located side by side (insert, Figure 3C). The number of BrdU-positive cells per square millimeter in the prelimbic cortex of the different treatment groups is shown in Figure 3D [control, 4.10 ± .79; fluoxetine 5 mg/kg, 7.57 ± .36; olanzapine 2 mg/kg, 6.24 ± .74; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 7.23 ± .27; n = 5 per group; $F(3,15) = 7.033$; p = .0036].

Chronic administration of fluoxetine alone significantly increased the density of BrdU-positive cells in the prelimbic cortex, 185% relative to vehicle (p = .0007, Fisher's PLSD). Furthermore, olanzapine alone also enhanced the density of BrdU-positive cells, by 156% (p = .0195, Fisher's PLSD). The effect of the combination of fluoxetine and olanzapine did not differ from the

fluoxetine-alone group. The number of BrdU-positive cells per square millimeter in the primary motor cortex is shown in Figure 3E (control, 4.04 ± .90; fluoxetine 5 mg/kg, 4.25 ± .32; olanzapine 2 mg/kg, 3.83 ± .28; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 4.36 ± .31; $n = 5$ per group). There was no significant difference among the four treatment groups in this region. This suggests that the increase in the density of BrdU-positive cells in the prelimbic cortex is specific to this brain area; however, the area analyzed in these two regions contains different layers of cortex, owing to the structure of prelimbic cortex and the size of the counting area. Prelimbic cortex includes layers I–VI, whereas motor cortex includes layers I–IV. It is possible that this could influence the outcome in the motor cortex, and this must be examined in future studies.

To determine whether cell proliferation in other brain regions is affected by fluoxetine or olanzapine, the striatum and subventriclar zone were examined. Striatum is another brain region where a reduction in volume has been reported in clinical imaging studies (Krishnan et al 1992; Parashos et al 1998), and the subventricular zone is the other major neurogenic zone of the adult brain. Representative BrdU immunohistochemical stained sections of the striatum are shown in Figures 4B and C. The numbers of BrdU-positive cells per square millimeter of the striatum are shown in Figure 4F (control, 3.23 ± .67; fluoxetine 5 mg/kg, 3.49 ± .31; olanzapine 2 mg/kg, 4.24 ± .87; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 4.30 ± 1.20; $n = 5$ per group). There was no significant difference between the four groups tested.

Representative sections of BrdU immunohistochemistry of the subventricular zone are shown in Figure 4D and E. The density of BrdU-positive cells in the subventricular zone is much greater than in any of the other brain regions examined, including the hippocampus. The number of BrdU-positive cells per square millimeter in the subventricular zone is shown in Figure 4G (control, 31.16 ± 4.38; fluoxetine 5 mg/kg, 28.83 ± 2.78; olanzapine 2 mg/kg, 31.02 ± 1.68; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 31.10 ± 1.76; $n = 5$ per group). As was observed in the striatum, there was no significant difference in the number of BrdU-labeled cells in the subventricular zone. This is consistent with a previous a report from this laboratory on the actions of fluoxetine (Malberg et al 2000).

Influence of Subchronic Olanzapine and Fluoxetine on Cell Proliferation in the Dentate Gyrus and Prelimbic Cortex

To determine whether the combination of fluoxetine and olanzapine has a more rapid effect on cell proliferation than either treatment alone, the influence of 1-week coadministration was examined (Figure 5). The number of BrdU-positive cells in the dentate gyrus was not significantly different between the fluoxetine or olanzapine-alone groups and the control group. There was also no significant effect of the combination of fluoxetine plus olanzapine (control, 6620 ± 27; fluoxetine 5 mg/kg, 6110 ± 37; olanzapine 2 mg/kg, 6900 ± 53; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 6830 ± 28; $n = 6$ per group) (Figure 5A).

In the prelimbic cortex, where chronic treatment with either fluoxetine or olanzapine enhanced cell proliferation, there was also no difference among four groups after subchronic treatment (BrdU-positive cells per square millimeter: control, 5.59 ± .32; fluoxetine 5 mg/kg, 6.59 ± .72; olanzapine 2 mg/kg, 7.16 ± .71; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 7.09 ± .84; $n = 6$ per group) (Figure 5B).

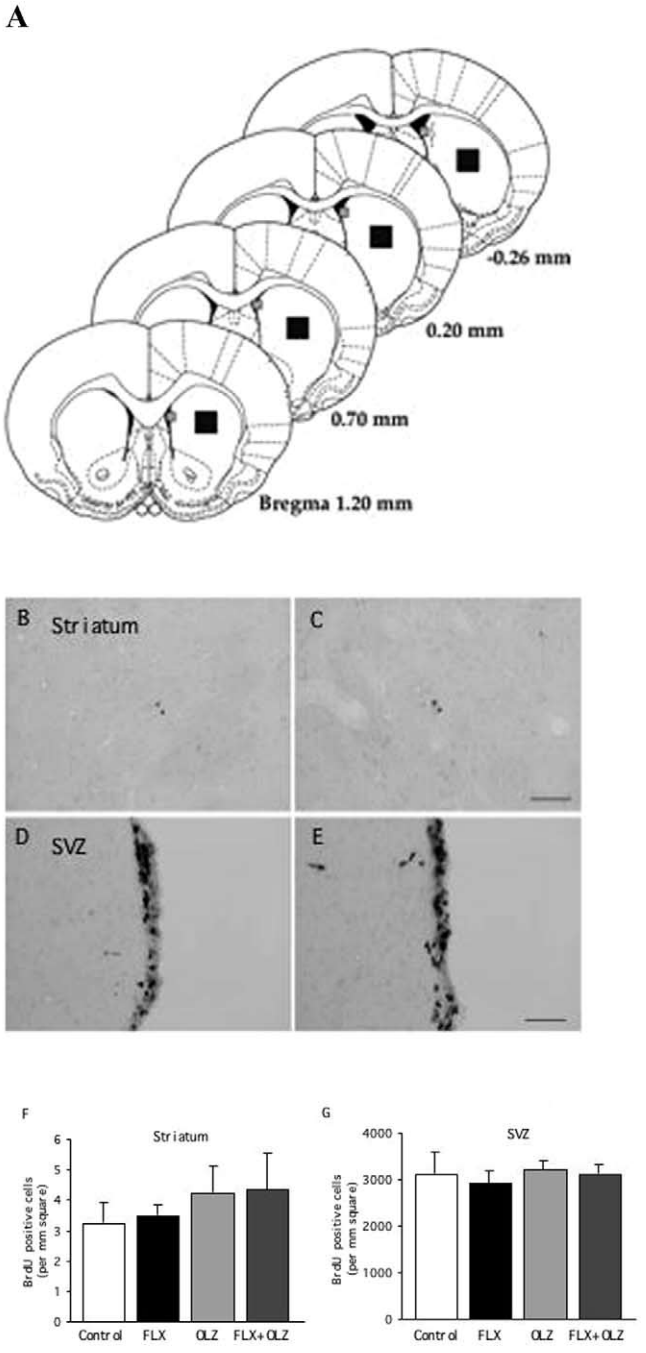

Figure 4. Chronic administration of olanzapine (OLZ) or fluoxetine (FLX) does not influence the number of bromodeoxyuridine (BrdU)-labeled cells in the subventricular zone (SVZ) or the striatum of rat brain. Rats were administered FLX (5 mg/kg), OLZ (2.0 mg/kg), or the combination of FLX + OLZ (5.0 and .5 mg/kg, respectively) for 21 days. Rats were then administered BrdU (150 mg/kg) 3 hours after the last drug treatment and killed 24 hours later for BrdU immunohistochemistry. **(A)** Regions of interest on a series of plates from Paxinos and Watson 1997. The black square indicates the striatum **(B, C, F)** (1 × 1 mm) and the gray square indicates the SVZ of the lateral ventricle **(D, E, G)** (.1 × .1 mm). **(B–E)** Representative BrdU-labeled sections of the **(B, C)** striatum and (D, E) SVZ. Scale bar = 50 μm. The number of BrdU-positive cells was determined by stereologic analysis throughout the regions indicated in **A**, and the results are expressed as the number of cells per square millimeter for **(F)** striatum and **(G)** SVZ. Values are expressed as mean ± SEM. Results were subjected to statistical analysis. *$p < .05$ (analysis of variance and Fisher's post hoc test).

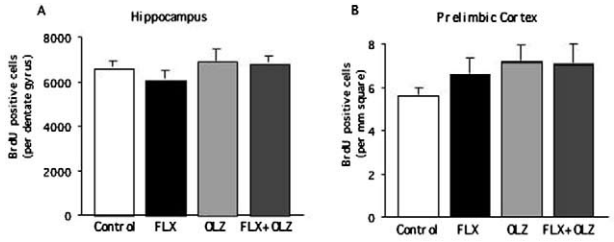

Figure 5. Subchronic (1 week) administration of fluoxetine (FLX) or olanzapine (OLZ) does not influence the number of bromodeoxyuridine (BrdU)-labeled cells in the hippocampus or prelimbic cortex. Rats were administered FLX and OLZ and then BrdU exactly as described in Figures 2–4 and Methods and Materials. The number of BrdU-positive cells was determined in **(A)** the dentate gyrus of the hippocampus and **(B)** the prelimbic cortex. Rats were killed 24 hours after BrdU injection. Values are expressed as **(A)** the total numbers of cells per hippocampus or **(B)** per square millimeter of the area quantified in the prelimbic cortex and are the mean ± SEM. Results were subjected to statistical analysis. *$p < .05$ (analysis of variance and Fisher's posthoc test).

Influence of Olanzapine and Fluoxetine Administration on the Survival of BrdU-Labeled Cells

To determine whether the newborn cells in the dentate gyrus and prelimbic cortex survive after chronic fluoxetine or olanzapine administration, the number of BrdU-positive cells was examined 28 days after drug treatment and BrdU administration. The effect in the dentate gyrus is shown in Figure 6A [control, 6730 ± 720; fluoxetine 5 mg/kg, 9590 ± 630; olanzapine 2 mg/kg, 9040 ± 600; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 9630 ± 1070; $n = 6$ per group; $F(3,20) = 3.122$; $p = .0489$]. Fluoxetine or olanzapine alone significantly increased the number of surviving cells in the subgranular zone ($p = .0169$ and .0481, respectively, Fisher's PLSD). Although the combination of fluoxetine and olanzapine increased the BrdU-positive cells compared with the control group ($p = .0157$, Fisher's PLSD), it did not increase the cell survival to a greater extent than either treatment alone. These findings are consistent with the results observed 24 hours after BrdU administration, indicating that the newborn cells survive for at least 28 days after proliferation.

Figure 6B shows the number of BrdU-positive cells in the prelimbic cortex 28 days after drug and BrdU treatment. In contrast to the dentate gyrus, there was no difference among the four groups in this region (control, 11.19 ± 1.18; fluoxetine 5 mg/kg, 10.69 ± 1.14; olanzapine 2 mg/kg, 11.39 ± .70; fluoxetine 5 mg/kg + olanzapine .5 mg/kg, 11.62 ± .87; $n = 6$ per group). This means that the effect of increased proliferation that was seen immediately after chronic fluoxetine and olanzapine administration was reversed after 28 days.

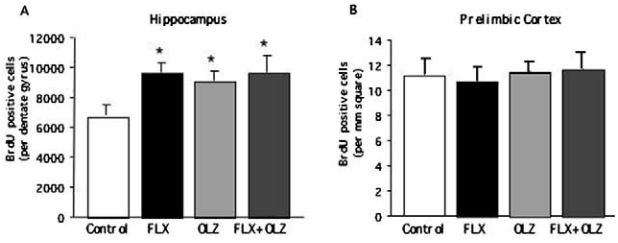

Figure 6. Effect of chronic fluoxetine (FLX) and/or olanzapine (OLZ) treatment on the survival of bromodeoxyuridine (BrdU)-positive cells in the **(A)** dentate gyrus and **(B)** prelimbic cortex. Rats were administered FLX and/or OLZ, exactly as described in Figures 2–4 and Methods and Materials. For this experiment, animals received BrdU (100 mg/kg) for the last three drug treatment days and were killed 28 days after the last injection. The number of BrdU-positive cells was determined in the dentate gyrus of the **(A)** hippocampus or **(B)** prelimbic cortex. Values are expressed as **(A)** the total numbers of cells per hippocampus or **(B)** per square millimeter of the area quantified in the prelimbic cortex and are the mean ± SEM. Results were subjected to statistical analysis. *$p < .05$ (analysis of variance and Fisher's post hoc test).

Characterization of the Phenotype of BrdU-Labeled Cells

To determine the phenotype of newborn cells in the dentate gyrus granule cell layer, colabeling with a neuronal marker (NeuN), an astrocyte marker (S100β), and BrdU was conducted. Representative sections that were triple-labeled with anti-NeuN, S100β, and BrdU antisera are shown in Figure 7. The majority of BrdU-positive cells in the granule cell layer of dentate gyrus were colabeled with NeuN, consistent with previous reports (Malberg et al 2000; Nakagawa et al 2002). The effect of chronic drug treatment on the phenotype of BrdU-positive cells is shown in Table 1. Please note that the phenotyping was of cells located in the dentate gyrus granule cell layer where there is a higher percentage of neurons than in the hilus (Malberg et al 2000). The percent of NeuN or S100β and BrdU double-labeled cells was not changed by either fluoxetine or olanzapine or by coadministration of these two drugs.

We also determined the phenotype of the BrdU-positive cells in the prelimbic cortex by triple-labeling with anti-NeuN and anti-S100β antibodies (Figure 7 E-H). No BrdU positive cells were colabeled with NeuN, indicating that there are no new neurons in the cerebral cortex. BrdU positive cells were often observed close to or touching NeuN positive cells, as previously reported (Gould et al 2001). Very few BrdU positive cells were colabeled with S100β (Table 1). Only three S100β and BrdU double-labeled cells were observed in a total of 607 BrdU positive cells examined in all four treatment groups. Double labeling was also conducted with a marker for oligodendrocytes (O4) or endothelial cells (RECA-1) and BrdU (Figure 7I and 7J). Few BrdU-positive cells expressed the oligodendrocyte marker (Table 1), with only 4 out of 616 BrdU-positive cells in all groups demonstrating colabeling in the prelimbic cortex. In contrast, there was a higher number (approximately 20%) of BrdU-positive cells that were colabeled with the RECA-1 antibody. The number of RECA-1/BrdU doubled-labeled cells was not altered by chronic drug treatment (Table 1).

Discussion

The results of this study extend previous work on the regulation of cell proliferation by antidepressant treatments with two significant findings. First, the results demonstrate that an atypical antipsychotic drug used alone or for augmentation therapy of depressed patients increases neurogenesis in the adult hippocampus, similar to antidepressant drug treatments. Second, the results show that administration of an SSRI or an atypical antipsychotic increases cell proliferation in the prelimbic cortex, a region where reduced volume and decreased cell number have been reported in brain imaging and postmortem studies of depressed patients.

Olanzapine and Increased Neurogenesis in the Hippocampus

The upregulation of neurogenesis in the adult hippocampus in response to olanzapine was observed after chronic (21 days) but not subchronic (7 days) administration, consistent with the time course for fluoxetine in the current study and other antidepressants in previous reports (Malberg et al 2000; Manev et al 2001; Santarelli et al 2003). Coadministration of fluoxetine and

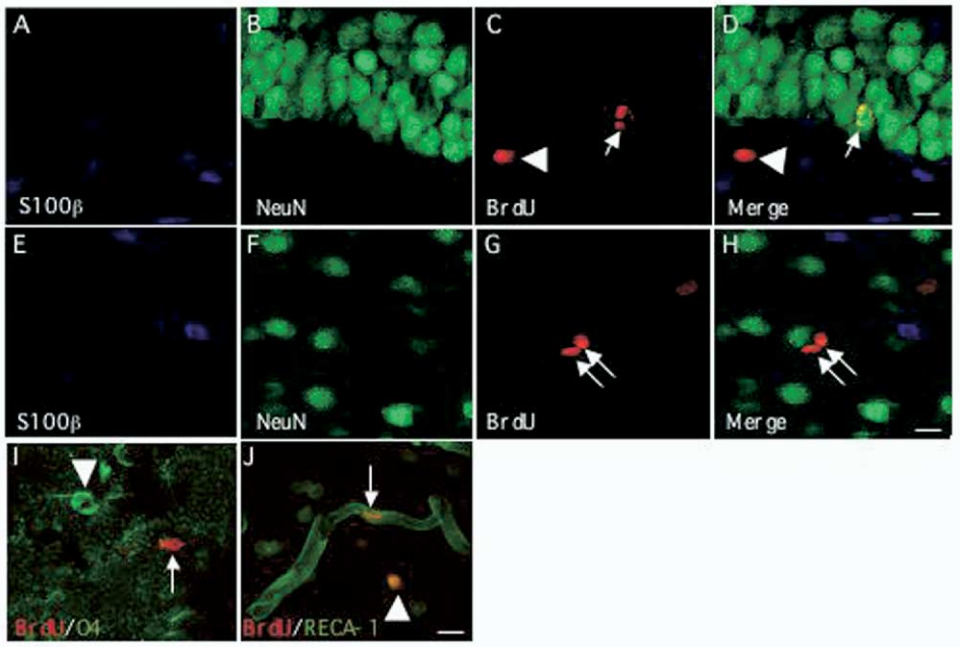

Figure 7. Characterization of the phenotype of bromodeoxyuridine (BrdU)-positive cells in the hippocampus and prefrontal cortex. Rats were administered fluoxetine (FLX) and olanzapine (OLZ) exactly as described in Figures 2–4 and Methods and Materials. For this experiment, animals received BrdU (100 mg/kg) for the last three drug treatment days and were killed 28 days after the last injection. Sections from hippocampus **(A–D)** or prefrontal cortex **(E–J)** were subjected to immunohistochemical analysis with phenotypic markers for glia (S100β), neurons (NeuN), oligodendrocytes (04), and endothelial cells (RECA-1), as well as BrdU. Representative confocal micrographs are shown for labeling of **(A, E)** S100β (blue), **(B, F)** NeuN (green), and **(C, G)** BrdU (red), as well as the merge of all three labels **(D, H)**. In the hippocampus, an arrow indicates a BrdU(+)/NeuN(+)/S100β(−) cell in the subgranular zone, and an arrowhead points to a BrdU(+)/NeuN(−)/S100β(−) cell in the hilus **(C, D)**. In the prefrontal cortex, arrows indicate BrdU-positive cells close to a NeuN-positive cell **(G, H)**. **(I)** A representative section of the prefrontal cortex that is double-labeled for the oligodendrocyte marker O4 (green) and BrdU (red). Arrow indicates a BrdU(+)/O4(−) cell; arrowhead points to an O4(+) cell. **(J)** A representative section of the prefrontal cortex double-labeled for the endothelial cell marker RECA-1 (green) and BrdU (red). Arrow indicates a BrdU(+)/RECA-1(+) cell; arrowhead points to a BrdU(+)/RECA-1(−) cell. Quantitative analysis of the percentage of cells expressing a specific phenotypic marker and BrdU was conducted, and the results are shown in Table 1. Scale bar = 10 μm.

olanzapine did not result in a greater upregulation of neurogenesis in the hippocampus than that observed with either drug alone, suggesting that these two agents might be acting through a similar downstream mechanism. The combination of these two drugs also did not result in a more rapid induction of neurogenesis after 7 days of administration. Olanzapine administration did not significantly influence the percent of neurons and glia in the hippocampus, as observed with fluoxetine and other types of antidepressants (Malberg et al 2000).

Previous studies on the regulation of neurogenesis by typical and atypical antipsychotic drugs have been mixed. Studies of a typical antipsychotic, haloperidol, report that a low to moderate dose does not influence neurogenesis in the adult hippocampus (Halim et al 2004; Malberg et al 2000; Wakade et al 2002; Wang et al 2004) (Table 2). Another study has reported that haloperidol administration increases the number of BrdU-positive cells in the dentate gyrus, although the dose of haloperidol was higher (5 mg/kg), gerbils were used instead of rats, and the dose of BrdU was lower (50 mg/kg) (Dawirs et al 1998) (see Cameron and McKay 2001 for cell labeling with different doses of BrdU). A study of atypical antipsychotics (olanzapine and risperidone) reported a small but nonsignificant increase in the number of BrdU-labeled cells in the hippocampus (Wakade et al 2002). The numbers of animals tested ($n = 3$) was relatively small and was insufficient for statistical significance. Two reports published since the manuscript for this report was submitted demonstrate that chronic olanzapine (Wang et al 2004) or clozapine (Halim et al 2004) treatment have similar effects on cell proliferation in the dentate gyrus, although the dose used for olanzapine (10 mg/kg) was substantially higher than that used in the current study.

The molecular mechanisms underlying the upregulation of neurogenesis in hippocampus by olanzapine are not known. Olanzapine and other atypical antidepressants are reported to be functional agonists of 5-HT_{1A} receptors and to antagonize 5-HT_{2A}

Table 1. Percentages of Colocalization of Phenotypic Markers with Bromodeoxyuridine

	Dentate Gyrus		Prelimbic Cortex			
	NeuN	S100β	NeuN	S100β	O4	RECA-1
Control	87.9	1.01	0	0	0	18.2
Fluoxetine	88.5	1.01	0	.67	.66	16.7
Olanzapine	88.7	.67	0	1.33	.64	18.2
Fluoxetine + Olanzapine	93.3	1.33	0	0	1.92	14.1

NeuN, mouse antineuronal nuclei; RECA-1, mouse antirat endothelial cell antigen-1.

Table 2. Summary of the Effects of Chronic Selective Serotonin Reuptahe Inhibitor (SSRI) Antidepressant and Atypical or Typical Antipsychotic drug (APD) Treatments on Cell Proliferation in Different Brain Regions

Brain Region	SSRI	Atypical APD	Typical APD
Hippocampus	Increased[a,b]	Increased[a,c,d]	No effect[b,c,d,e]
Prefrontal Cortex	Increased[a]	Increased[a,c]	No effect[c]
Motor Cortex	No effect[a]	No effect[a]	Not tested
Striatum	No effect[a]	Increased[c]/trend[a]	No effect[c]
Subventricular Zone	No effect[a,b]	No effect[a]/increased[b]	No effect[b]

The results presented in this table are based on the current study and recent reports. Each study examined specific classes of drugs and brain regions, as indicated. The SSRI tested in these studies is fluoxetine. The atypical APDs tested are one or more of the following: olanzapine, clozapine, or risperidone and the typical APD is haloperidol at the doses indicated for each study below.

[a]Present study (tested olanzapine [.5 and 2.0 mg/kg] and fluoxetine [5 mg/kg] for 21 days).

[b]Malberg et al 2000 (tested fluxetine [5 mg/kg, 14 and 28 days] and haloperidol [1 mg/kg for 7 days and 2 mg/kg for the next 7 days]).

[c]Wang et al 2004 (tested olanzapine [10 mg/kg] haloperidol [2mg/kg] for 21 d).

[d]Halim et al 2004 (tested clozapine [.5 and 20 mg/kg] and haloperidol [.5 and 2.0 mg/kg] for 28 days).

[e]Wakade et al 2002 (tested olanzapine [2.0 mg/kg], risperidone [.5 mg/kg], and haloperidol [.4 mg/kg] for 21 days).

receptors (see Marek et al 2003; Meltzer et al 2003). Both of these receptors have been positively linked to the regulation of neurogenesis in the hippocampus (Banasr et al 2004; Santarelli et al 2003). Atypical antipsychotic drugs are also reported to influence growth factor systems in the brain, including nerve growth factor and fibroblast growth factor 2 and could influence hippocampal neurogenesis in this manner (Li et al 1999; Riva et al 1999).

The effects of olanzapine on hippocampal neurogenesis are similar to the actions of fluoxetine, an SSRI, as well as several other classes of antidepressants, including norepinephrine selective reuptake inhibitors, monoamine oxidase inhibitors, and electroconvulsive seizures (Madsen et al 2000; Malberg et al 2000; Manev et al 2001; Santarelli et al 2003; see Duman 2004). A recent study has demonstrated that blockade of neurogenesis by focused irradiation or use of a mutant mouse model blocks the effects of antidepressants in two behavioral models, novelty suppressed feeding and chronic mild stress (Santarelli et al 2003). Increased hippocampal neurogenesis could increase certain aspects of hippocampal function relevant to mood disorders, including control of the hypothalamic–pituitary–adrenal axis, cognitive function, and anxiety (Duman 2004; Santarelli et al 2003). These findings are consistent with the possibility that the actions of olanzapine are mediated in part by regulation of neurogenesis, and further studies will be required to test this hypothesis.

Atypical but not typical antipsychotic drugs are reported to produce beneficial effects in depressed patients (Ghaemi and Goodwin 1999; Rothchild 2003; Sajatovic et al 2002). The potential clinical relevance of altered neurogenesis in the hippocampus is suggested by brain imaging studies demonstrating that there is a reduction in the volume of hippocampus in patients with depression or PTSD (Bremner et al 1995; Frodl et al 2002; MacQueen et al 2003; Mervaala et al 2000; Shah et al 1998; Sheline et al 1996, 1999; Steffens et al 2000). In addition, the reduction in hippocampal volume is inversely proportional to the amount of time a patient is medicated with an antidepressant (Sheline et al 2003), and reduced hippocampal volume is partially reversed after antidepressant treatment (Vermetten et al 2003). It is likely that other morphologic effects, such as regulation of neuropil or dendrite arborization, contribute to the regulation of hippocampal volume, but regulation of neurogenesis could be a contributing factor (Czeh et al 2001; Duman 2004; McEwen 1999). On the basis of the results of the present study, it would be interesting to test the influence of an atypical antipsychotic on the volume of hippocampus in depressed patients.

Increased Cell Proliferation in the Prelimbic Cortex

The second major finding of this study is that olanzapine or fluoxetine administration increases the number of BrdU-labeled cells in the prelimbic cortex. We have also found that electroconvulsive seizure administration increases the number of BrdU-labeled cells in the prefrontal cortex (Madsen et al, in press). As was observed in the hippocampus, increased cell proliferation in the prelimbic cortex was dependent on chronic drug treatment. This effect is consistent with a report (Wang et al 2004) published since the submission of this article in manuscript form (see Table 2). There was no effect of either olanzapine or fluoxetine on the number of BrdU-labeled cells in the primary motor cortex or subventricular zone. Another study has reported that chronic olanzapine administration increases the number of BrdU-labeled cells in the subventricular zone (Wakade et al 2002). The reason for the discrepancy between this previous study and the current report is not clear but could be due to a difference in the subregion of subventricular zone examined or the dose of BrdU administered (50 vs. 150 mg/kg). In the striatum, there was a tendency for an increase in the number of BrdU-positive cells that is similar in magnitude to that in hippocampus. This effect is consistent with the highly significant and robust induction of cell proliferation reported in a recent study (Wang et al 2004), and the greater increase could be due to the higher dose of olanzapine used (10 mg/kg) relative to the current study (2 mg/kg).

In the current study, we found that the combination of olanzapine plus fluoxetine did not produce a greater increase in the number of BrdU-positive cells than either drug alone. This suggests that fluoxetine alone would be sufficient to produce a maximum response and therefore could not account for the augmentation that has been observed clinically; however, the clinical approach has been to add olanzapine after a patient has failed to respond to an SSRI like fluoxetine. This was the case in a study in which olanzapine was added after 6 weeks of fluoxetine treatment (Shelton et al 2001). There are several potential explanations for this interaction. One possibility is that olanzapine and fluoxetine have different proximal effects (e.g., blockade of 5-HT_{2A} receptors and 5-HT transporter, respectively) but have the same downstream or distal effects (e.g., regulation of intracellular signaling and cell proliferation). If for some

reason 5-HT neurotransmission is functioning improperly in a subgroup of depressed patients, then olanzapine could increase cell proliferation by circumventing the proximal target used by an SSRI. Another possibility is that olanzapine could act by opposing the effects of stress or depression and might not have an effect in normal animals. For example, it is possible that olanzapine blockade of 5-HT_2 receptors has little effect under normal conditions but blocks the effects of stress on neurogenesis.

Analysis of the phenotype of the BrdU-labeled cells in the prelimbic cortex was conducted with a number of different cell markers. Triple-labeling studies demonstrate that the BrdU-labeled cells do not express markers of neurons (NeuN) or astrocytes (S100β). There has been controversy regarding the birth of new neurons in the adult cerebral cortex, with reports demonstrating the presence (Altman 1963; Bernier et al 2002; Gould et al 1999, 2001; Kaplan 1981) and absence (Koketsu et al 2003; Kornack and Rakic 2001; Korr et al 1973; Wang et al 2004; Madsen et al, in press) of sustained neurogenesis. The discrepancy between these studies could be due to methodologic issues, such as the dose of BrdU, tissue freezing, and staining protocols. There are also reports that insults that cause cell death can induce neurogenesis in the adult cerebral cortex (Arvidsson et al 2002; Magavi et al 2000). In the present study, many of the BrdU-labeled cells (approximately 40%) were closely apposed to a NeuN-positive cell, as previously reported, and are referred to as satellite cells (Gould et al 2001). These cells were not labeled with S100β. In fact, there were very few cells colabeled with either S100β or the oligodendrocyte marker (04) (Table 1), consistent with a previous study of the prefrontal cortex of adult macaque monkeys (Koketsu et al 2003). There is one earlier report demonstrating that trauma induces newborn oligodendrocytes in adult brain (Ludwin 1984), and we have found colocalization of another oligodendrocyte marker (Rip) with BrdU (Madsen et al, in press); however, we could not obtain significant staining with the Rip antibody in the present study. The reason for the discrepancy with the current and previous report (Madsen et al, unpublished data) is not clear but could be related to a very narrow titer curve for specific staining or the batch of antibody used for this study.

We have also examined the expression of an endothelial cell marker, RECA-1, and found that approximately 20% of the BrdU-positive cells in the prelimbic cortex were colabeled with this marker. This is consistent with previous reports demonstrating that newborn cells in the cerebral cortex express endothelial cell markers (Korr et al 1973; Mares and Bruckner 1978; Madsen et al, in press). The percentage of BrdU and RECA-1 double-labeled cells was the same in the prelimbic regions of vehicle- and drug-treated animals, indicating that there was no shift in cell differentiation (Table 1). There are a number of factors that are known to influence the formation of endothelial cells and angiogenesis, including vascular endothelial growth factor, fibroblast growth factor-2, and platelet-derived growth factor (Jin et al 2002; Newton et al 2003; Raballo et al 2000). Many of these factors are also reported to influence neurogenesis, to further complicate the picture. The role of these factors in the actions of atypical antipsychotic and antidepressant drugs remains to be determined.

In contrast to the dentate gyrus, there was no increase in levels of BrdU-positive cells 4 weeks after BrdU administration, indicating that the cells that were induced to proliferate in the prelimbic cortex in response to fluoxetine or olanzapine treatment are not stable. Another possibility is that the newborn cells in this region continue to divide, which would result a dilution of BrdU incorporated during the earlier proliferation phase. In either case, it is possible that the newborn cells serve a short-term function and are not required to survive for long periods. Alternatively, it is possible that continued administration of olanzapine or fluoxetine treatment would result in sustained survival of newborn cells in the prelimbic cortex. Wang et al (2004) found a significant increase in the number of BrdU-labeled cells 2 weeks after BrdU administration, regardless of whether olanzapine treatment was continued. It is also possible that the higher dose (10 mg/kg) of olanzapine used in the latter study could have a more prolonged effect on the survival of newborn cells.

Electroconvulsive seizure treatment is also reported to increase the number of endothelial cells in the hippocampus (Hellsten et al 2004) and prefrontal cortex (Madsen et al, in press). In the hippocampus, endothelial cell proliferation and neurogenesis occur in close conjunction, leading to the hypothesis of a functional link, referred to as the "vascular niche" (Palmer et al 2000). There was no association of endothelial cells, either existing or newborn, with other BrdU-positive cells, in the prelimbic cortex, indicating that the vascular supply is sufficient for the cell proliferation in this brain region. The functional significance of increased endothelial cell proliferation is not clear, but there is evidence that electroconvulsive seizures increase a number of angiogenic factors, raising the hypothesis that increased angiogenesis contributes to the actions of antidepressant treatment (Newton et al 2003).

Brain imaging and postmortem studies support the possibility that altered cell proliferation occurs in depression. Imaging studies have reported that the volume of the prefrontal cortex is reduced in patients with depression or bipolar disorder (Drevets et al 1997). Postmortem studies from several different groups report a decrease in the density of glia and the size of neuronal cell bodies in the subgenual, dorsal lateral prefrontal, and cingulate cortex (Cotter et al 2001, 2002; Ongur et al 1998; Rajkowska et al 1999). It is possible that a decrease in glia as well as vascular support contributes to the decrease in neuronal body size observed in postmortem studies of depressed patients. The results of the current study are consistent with the hypothesis that upregulation of cell proliferation in the prelimbic cortex could block or reverse the decrease in the number of glia observed in depressed patients. Studies are currently underway to determine whether exposure to stress or behavioral despair in models of depression decrease cell proliferation in the prelimbic cortex.

Decreased glia and reduced neuronal body size could influence the function of the prefrontal cortex, which plays an important role in cognitive function, such as working memory. Atypical but not typical antipsychotic agents are reported to improve cognitive deficits in schizophrenic patients (Harvey et al 2003; Kasper and Resinger 2003; Purdon et al 2000). Depressed patients also exhibit cognitive difficulties, and atypical antipsychotics could serve to alleviate this symptom of depression.

The results of this study extend previous reports on antidepressant regulation of neurogenesis in adult hippocampus to antidepressant and antipsychotic drug regulation of cell proliferation in the adult prelimbic cortex. Additional studies will be required to identify the mechanisms underlying the regulation of cell proliferation in these two regions and to determine the interaction of antidepressants and stress. Moreover, functional studies are required to determine the significance of cell proliferation in both hippocampus and prelimbic cortex with regard to the behavioral and antidepressant effects of fluoxetine and

olanzapine, and to determine whether these effects generalize to other SSRI and atypical agents. Finally, the results further demonstrate that chronic administration of psychotropic drugs used for the treatment of mood disorders results in cellular alterations in limbic brain regions.

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